YANA VAN DYKE

Practical Applications of Protease Enzymes in Paper Conservation

ABSTRACT

Paper conservators have long recognized the benefits of enzymes in the conservation treatment of works of art. Most commonly, hydrolase-type enzymes are employed in the conservation of works on paper to assist in the breakdown of adhesive residues from previous restorations or to facilitate the removal of secondary supports such as linings or mounts. The principal advantages of these enzymes are their specificity and efficiency in catalyzing hydrolytic cleavage of polymers such as proteins, polysaccharides, and lipids. The author evaluates the effectiveness of two protease enzymes while taking into consideration cost effectiveness, optimal working conditions, and after-treatment effects. The fruition of this research was realized in the successful conservation treatment of an Indian miniature utilizing the protease enzyme trypsin incorporated into an agarose gel. The conservation treatment of this item is discussed in detail.

INTRODUCTION

Enzymes make up the largest and most highly specialized class of protein molecules. These complex proteins, produced from living cells, are the primary instruments for the expression of gene action since they catalyze thousands of biochemical reactions. These large globular proteins act as catalysts for biochemical reactions, providing the lower-energy pathway between reactants and products.

Like all proteins, enzymes are composed mainly of the twenty naturally occurring amino acids. Amino acids link together to form a polypeptide backbone, creating macromolecules that fold into three-dimensional conformations to facilitate catalysis. Individual amino acid side chains supply chemical reactivity of different types that are exploited by the enzyme in catalyzing specific chemical transformations. What distinguishes each amino acid chemically and physically is the identity of the side chain. Chemical properties of the side chains include hydrophobicity, hydrogen bonding, salt bridge formation, amino acids acting as acids and bases, cationic metal binding, covalent bond formation, and disulfide bridging, among others (fig. 1).

Functioning spontaneously outside of the cell, these proteins lend themselves to laboratory experiments. In the last twenty years, we have witnessed an unprecedented expansion in our understanding and use of enzymes in a broad range of research and industrial applications. Today we are in the golden age of enzyme technology. Beyond their applications in commercial industries related to food, textile, leather, pulp, and paper, enzymes are the focus of intense research within the biomedical community. Scientists found enzymes critical in sequencing DNA and mapping the human genome, and they are the driving force behind the therapeutic drug market for HIV drug-related cocktails. In addition, the U.S. Department of Defense has shown an interest in utilizing immobilized

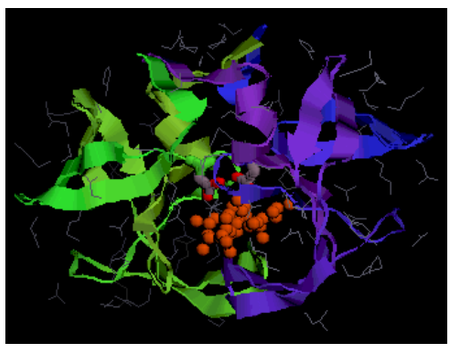

Fig. 1. Computer-generated model of a trypsin enzyme structure. The active site is in the center. From the ExPASy Molecular Biology Server (http://www.expasy.ch).

Presented at the Book & Paper Group Session, AIC 32nd Annual Meeting, June 9–14, 2004, Portland, Oregon. Received for publication Fall 2004. Color images for this paper are available online at http://aic.stanford.edu/bpg/annual/.

enzymes to develop decontamination systems against poisonous agents for homeland security and environmental protection (Pacific Northwest Laboratory 2002). One critical aspect of an enzyme's catalytic mechanism is how it brings together amino acid side chains that are distant from one another along the macromolecule's chain to create active centers. This process gives the enzyme molecule its overall size and shape in the binding cavity and also sets up the conditions for specific bond cleavage.

Within the enzyme collective is a group whose function is catalyzing hydrolytic cleavage of such biological polymers as proteins, polysaccharides, and lipids. These are known as hydrolases and are the type of enzyme most commonly used in paper conservation. Of all the types of enzyme-catalyzed reactions, hydrolytic transformations involving amide- and ester-bonds are the easiest to perform using proteases, esterases, or lipases. A large number of readily available enzymes possessing relaxed substrate specificities exist and are the main features that have made the hydrolases the favorite class of enzyme for organic chemists during the past decade. About two-thirds of the total research in the field of biotransformations has been performed using hydrolytic enzymes of this type and new discoveries have been transpiring at rapid rates in the last decade.

Two types of protease enzymes have been chosen for comparison in this investigation: trypsin and pepsin. The principal advantage of enzymes is their specificity, which enables only one kind of material, such as a starch or protein, to be rapidly acted upon. Each enzyme has specific bond-cleaving mechanisms and activity units. In order to evaluate the enzyme's effectiveness at removing a protein adhesive from a paper object, three types of papers were coated with a proteinaceous adhesive and subjected to enzyme immersion baths having the same concentrations of 200 units of activity per milliliter. The chosen papers were different in surface characteristics because the paper's topography may contribute to the retention of enzymes in the structure after treatment. The enzyme's speed of action and its potential for use depend, however, on a number of factors that may not be well defined for specific use in paper conservation.

HISTORY OF USE IN THE FIELD

Forty years ago, book conservator Paul Banks reported on the use of collagenase as a tool for the quick removal of animal glue accretions from paper artifacts. Since then, conservation professionals have recognized the beneficial uses of enzymes in the treatment of tenacious and intractable adhesives (Segal and Cooper 1977; Bansa and Hofer 1984). A thorough review on the history of enzyme use in paper conservation up until 1983 is found in Pia De Santis's article (1983). Many ambitious and forward-thinking investigations were undertaken in the field in the 1980s that studied enzymes and their viability in non-aqueous environments. Within the last fourteen years, two outstanding publications emerged on the use of *alpha*-amylase in paper conservation (Andrews 1990; Erickson 1992), but none has been published during that time on the specific uses of proteases. The most recent publication within the conservation literature on proteases was in 1987 (Grattan et al 1987). It outlined the types of enzymes important to paper conservators and reviewed their performance in various assays. This paper characterized four protease enzymes by examining the effects on the enzymes of temperature, pH, concentration, and shelf life, and evaluated their effectiveness at releasing adhesives.

The published conservation literature illustrates that historically experiments and treatments were limited to only two or three protease enzyme structures (Rickman 1988; Grattan et al. 1987): protease (bovine), protease (pancreatic crude), and *stryptomyces griseus*; all of which possess substantially low activities ranging from 1.1–13.8 units/mg. These enzymes were also somewhat expensive and resulted in commonly criticized high-cost conservation treatments.

Throughout the literature on paper conservation enzyme treatments there is great concern about the ability to rinse or denature the enzymes after treatment is completed. Common recommendations for removing or denaturing enzymes, appearing as early as 1977, include the use of hot water or alcohol baths (Segal and Cooper 1977). More recent studies report various rinsing procedures after employing an *alpha*-amylase enzyme and showed that the previously suggested denaturing steps were no more effective at removing enzymes than water rinsing alone (Erickson 1992; Andrews 1990). This is an important discovery because attempts to denature with hot water would be ineffective at the conventionally recommended temperatures (proteases reach their thermal optima in the range of 50°C–105°C), and because sufficiently elevated temperatures could pose serious risks to the art object being treated. One of these studies quantified the amount of residual enzymes left after water rinsing by using a radioisotope tagging system on the enzyme (Andrews 1990). The research showed that the residues were on the magnitude of 0.5 micrograms/cm^2 of paper or less than 2%.

The majority of the published research on enzymes describes treatment procedures as they relate to specific art objects (Hauser 1987; Shelley 1980; Wendelbo 1974; Grattan et al. 1987; van der Reyden 1988). One article addressed the effects of enzymes on a variety of media of a work of art on paper (Burgess and Charrette 1981). To date, only one partially published study has been found on the long-term effects of an enzyme treatment on a work of art on paper (Tse and Burgess 1988). This dearth shows the

relative gap in the literature for research related to the after-treatment effects of enzymes.

EXPERIMENTAL DESIGN

The following investigation was developed primarily to gain insight into practical conservation treatment procedures followed when using enzymes.[1] After researching which enzymes are needed, how do you to shop for them, and how do you interpret the plethora of enzymatic data available, develop methods of application, calculate concentrations, maintain pH and ion balance, and avoid any deleterious effects of the artworks being cared for? In addition to the need for treatment protocols, it is necessary to approach the question of whether enzyme treatments have any adverse effects on the artifact itself. Physical changes of the surrogate objects will be examined including: efficiency of removing the unwanted proteinaceous adhesive, possible retention of enzymes within the paper support, aging of these objects with possible enzyme residues, and subsequent color shifts, if any, produced by them.

The questions posed at the outset of this research were:

- Can a low-cost protease enzyme be found that is acceptable for use in paper conservation?
- Can a protease enzyme work at or close to room temperature, overriding the literature recommendations for working temperatures of 40°C–50°C?
- Does the surface character of the paper being treated affect the enzyme's ability to remove adhesives or the degree to which the enzyme is retained?
- Does the length of time of the rinse bath affect the overall appearance of the treated paper?
- Will there be a significant amount of enzyme residue left in the paper support if only water rinses are used? If so, will these residues change color over time?

Design

A comparison was made between the effectiveness of two protease enzymes: trypsin and pepsin. Three papers with different surface characteristics were brush-coated with two layers of an 8% solution of proteinaceous gelatin adhesive stained with the protein tagging chemical Rhodamine B isothiocyanate. The working procedure for the enzymes was by immersion. Enzymes with known activity rates at room temperature were selected. Each adhesive-coated sample was immersed in 10 ml of aqueous enzyme bath contained in a small, shallow, plastic tray. All enzyme baths had a concentration of 200 activity units per milliliter. Samples remained in their respective baths for a total time of thirty minutes.

Half of each of the pepsin- and trypsin-treated samples were rinsed in a bath of deionized water for half an hour, conditioned with the same buffering components as used in the enzyme immersion solution. The other halves were rinsed in two half-hour baths, totaling one hour rinse time. Under this protocol the rinse baths were refreshed after the first half hour. The samples were all dried in the same manner: placed between polyester web and blotters, placed under glass and weights, and allowed to dry for a period of seven days.

Following treatment and drying, the enzymes' effectiveness at removing the adhesive from the samples was judged and evaluated by a panel consisting of eight conservation specialists, with additional microscopic examination by the author. Both of these evaluation techniques resulted in assigned numerical codes for each sample, which were analyzed and averaged. Color measurements were also recorded before and after treatment with a Minolta chromameter. Variations on the effects of treatments relative to the surface characteristics of the paper were examined at this time and throughout the project. A portion of the sample population also underwent accelerated aging to track the changes that the retained residues exhibited. The aged samples were treated with the same evaluation procedures as noted above (fig. 2).

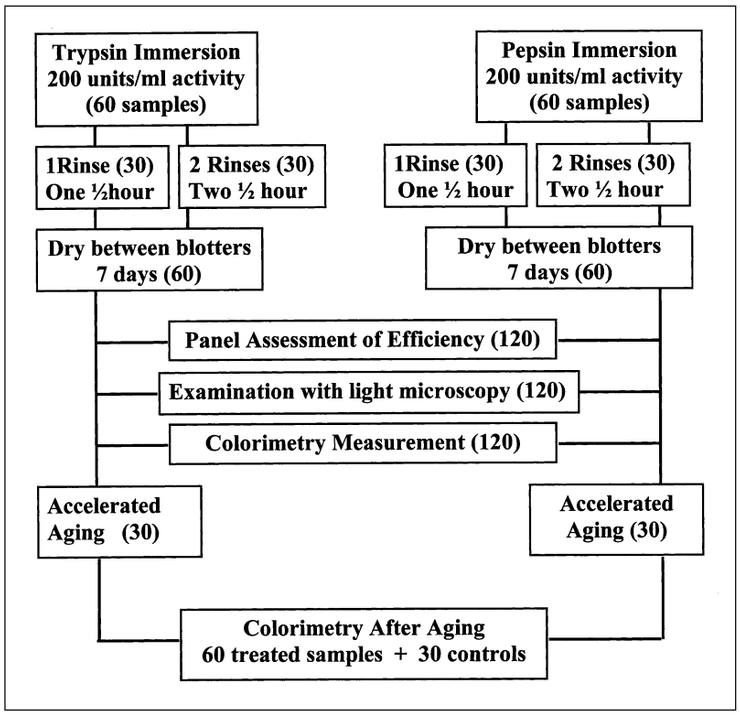

Fig. 2. Schematic of experimental samples: 120 samples, hot press, cold press, and rough-surfaced papers

Materials

Papers

The choice of the specific surrogate materials for the experiment was rationalized as follows: In paper conservation, the nature of the paper support can vary widely from a thin sheet to a thick sheet, and from a highly calendared paper to a rough textured paper based on the sheet's porosity. The three types of papers were chosen to represent a range of surface characteristics. The papers were: (1) a hot-pressed (smooth-textured) surface; (2) a cold-pressed (semi-textured) surface; and (3) rough, textured watercolor paper. All the papers were from T. H. Saunders, English papermakers, Waterford series, mould-made from 100% cotton, 90 lb./ream, neutral pH, four deckles, watermarked, white.

Gelatin

Collagen is the structural protein of connective tissue in animals and fish, found in various parts such as skin, muscle tissue, bone, and hide. Gelatin is the result of the separation of the triple-helical conformation of the protein collagen. Amino acid analysis of proteins used as adhesives show that the relative concentrations of the four major amino acids found in gelatin are 24.7% glycine (GLY), 13.0% proline (PRO), 9.7% glutamic acid (GLU), and 8.2% arginine (ARG) (Mills and White 1994).

Gelatin is produced by breaking collagen's strong interactive bond sites between the hydroxyl of the hydroxyproline and the amino hydrogens of the adjacent glycine units through extensive boiling. This partial denaturing of the collagen form of the protein makes gelatin soluble in water. Solutions of leached material with a concentration of more than 2%, such as gelatin, set on cooling and form very stiff gels with extremely powerful adhesive properties. Water-based solution adhesives become solidified through evaporation of the water and gain strength through secondary forces within the adhesive and between the adhesive and the adherend (the paper support).

High densities of polar groups from the paper's surface interact on a high-molecular-weight protein chain like gelatin to yield some of the strongest cohesions. Surface deviations of some papers, with varying degrees of amorphous or three-dimensional rough and open surface topography, make it possible for the protein adhesive to flow and penetrate into a relatively large surface area. In theory, rough surfaces have greater surface area and are better at interlocking than smooth surfaces. It is therefore thought that stronger bonds will be formed with the "rough" paper as opposed to the smooth and slick "hot press." Additionally questionable is whether cavities in the rough surface paper will make it more challenging for the enzyme to get in and clip at these adhesive-bonding sites.

The test papers were brush-coated twice with an 8% gelatin adhesive. In order to assist with visual assessment, colorimetry, and fluorescent densitometry evaluations, the gelatin was stained with the bright pink protein tagging chemical Rhodamine B isothiocyanate

Enzyme Classification

Categorized by the nature of the bond-cleavage reactions that they catalyze, enzymes are assigned numbers by the Enzyme Commission. In 1955, the International Union of Pure and Applied Chemistry formed the Enzyme Commission and began the enzyme nomenclature scheme. In its current edition, published by the International Union of Biochemistry and Molecular Biology, there are 12,796 different enzymes listed, compared to less than 3,000 just five years ago. The EC number begins with the broadest class, followed by a sequence of subclass numbers, defining the substrates and types of bonds upon which the enzyme acts more specifically within each.[2] Using trypsin as an example:

Trypsin 3.4.21.4

3	Hydrolases (enzymes that use water to break up some other molecule)
3.4	Peptidases (hydrolases that act on peptide bonds)
3.4.21	Serine endopeptidases (clipping from within the polypeptide chain)
3.4.21.4	Trypsin (from a multitude of sources: bacterial, various mammalian, etc.)

Enzyme Activity Units and Assays

The yardstick for enzyme performance is simply called a "unit," which is a defined amount of enzyme. A unit can be seen as a measure of speed: the more units, the faster a particular reaction will occur. Activities vary with temperature and pH, and test conditions are usually precisely defined as the assay. When purchasing enzymes, look for those with the most units per weight, which generally give the highest specific activity. Enzymes with a higher unit per weight also are the purest, requiring considerably less mass and lacking unwanted fillers, extenders, and bulking agents. Measuring the activity of an enzyme varies among research labs, but the Commission on Enzymes for the International Union of Biochemistry states that: "One unit (U) is defined as that amount of enzyme which will catalyze the transformation of one micromole of substrate per minute under defined conditions." Detection techniques for quantifying the loss of substrate or appearance of products that result from an enzyme are numerous and fall into six broad classes: spectroscopy, polarography, radioactive decay, electrophoretic separation, chromatographic separation, and immunological reactivity.

For investigators in different laboratories to reproduce one another's results, consistency in industrial applications, and a host of other critical reasons, it is imperative that product information and data be reported in meaningful units and be accompanied by sufficient details of the assay used. Standardized reporting of activity measurements is on its way to being universally adapted, and in general commercial manufacturers provide specifics on each lot of

enzyme isolated and sold in the consumer marketplace. Commonly provided are the specific buffer systems used in the reaction mixture, the pH and temperature at which the assay was recorded, the time interval over which initial velocity measurements were made, and the detection method used. Turnover numbers are typically reported as molarity change per unit time per molarity of enzyme; moles of substrate lost or product produced per unit time per mole of enzyme or equivalent; or molecules of substrate lost or product produced per unit time per molecule of enzyme.

Enzymes

The most abundant amino acids found in gelatin are glycine, proline, hydroxyproline, glutamic acid, and arginine. The Sigma Chemical catalog lists approximately fifteen different protease-type enzymes including chrymotrypsin, collagenase, elastase, and papain. While one may be able to find enzymes that have bond cleavage sites extremely specific to that of gelatin, the cost of these enzymes can be prohibitive in practically choosing them for a treatment. For example, one vial of metalloendopeptidase contains approximately only four units of activity and costs $175.

Two of the three principal digestive protease enzymes were selected for review: trypsin and pepsin. Trypsin is a pancreatic serine with substrate specificity based upon positively charged lysine and arginine side chains.[3] Pepsin, an acidic protease, is the principal proteolytic enzyme of vertebrate gastric juice; its specificity is broad range and it demonstrates an esterase activity.[4] The enzymes chosen belong to the group of hydrolases, which cleave the bonds of proteins, leading to shorter chain polypeptides and finally to water-soluble amino acid molecules. These two particular enzymes were also chosen for their relatively high activity, purity, and affordability.

1. Trypsin (EC 3.4.21.4). Type IX from Porcine Pancreas. T 0134. Crystallized, dialyzed, and lyophilized. Activity: 13,000–20,000 units per mg protein.
2. Pepsin (EC 3.4.23.1). P 6887. Crystallized and lyophilized. Activity: 3,200–4,500 units per mg protein

The most commonly purchased form of enzyme is a water-soluble lyophilized powder. The calculation for the number of grams of enzyme used was:

$$M = C \times V/A$$

where M = grams of enzyme; V = volume in milliliters of the enzyme solution having a concentration of C units of activity per milliliter of solution; and A = the activity of the solid.

The Sigma Chemical company provided specific activity of each enzyme lot. Trypsin 0134, (lot #076H1101) contained 15,200 units/mg of protein and Pepsin 6887 (lot #97H7465) contained 4,500 units/mg of protein. Therefore, the amounts of trypsin and pepsin needed for 10 ml and 200 units of activity/ml concentration were 0.00013 g per individual sample and 0.00044 g, respectively. However, a 750-ml volume of solution was made for ease of use as well as to keep exposure of packaged enzyme to air to a minimum during weighing.

Immersion Solutions and Conditions

Like all proteins, enzymes in their native states are optimally stabilized by specific solution conditions of pH, ionic strength, anion/cation composition, and so on. Generalities cannot be made with respect to these conditions and the best conditions for each enzyme must be determined empirically. Solution conditions for protein stability and optimum enzyme activity are not necessarily the same. In a conservation application, lack of cryogenic freezers and various other elements for optimal storage conditions limit enzyme purchases to an as-needed basis and may require that only enough solution is made for one day's work.

In most cases, the pH of an enzyme solution must be controlled in order to maintain or accomplish the activity of the enzyme in use. In fact, the use of a buffer to achieve pH-optimal conditions is recommended; however the dependency is largely "phenomenological" (Copeland 2000). Some enzymes have optimum efficiency at a specific pH, while other enzymes exhibit a biphasic behavior in that they operate best at two distinct pH optima. An enzyme solution that is not buffered to maintain the optimum pH might be effective but the action will be slower or may not occur at all.[5]

The direct bearing of pH on an enzyme's effectiveness was observed especially in the case of Pepsin 6887. Initial trials were run with a solution that consisted of deionized water, enough pepsin for an activity of 200 units/ml, and 0.05% potassium phosphate that buffered the solution at pH 5.5. At this pH, Pepsin 6887 was not active or extremely sluggish.

Various alterations were made to the enzyme solution in hopes of resolving pepsin's lack of activity. Altering the concentration of the enzyme, adding a bile acid (thinking that a bile acid/surfactant was needed to combat the hydrophobic nature of the alkyl-ketene dimer sizing agent in the paper), increasing immersion times, and lowering the pH with citric acid were all attempted. In the end, the pH proved to be the most influential element for induction of optimum activity for pepsin. The final working solution consisted of deionized water, 0.5% of potassium phosphate, 0.5% of citric acid, and 0.4316 g of Pepsin 6887 (200 activity units/ml). This solution was held at a constant pH of 3.4 throughout the trial.

Final working solutions with trypsin consisted of 750 ml of deionized water, 0.01315 g of Trypsin 0134 (200 activity units/ml), and enough calcium hydroxide to maintain a pH of 7.5. The presence of Ca^{2+} (20 mM) also served to retard trypsin's ability (inherent vice) to self-

digest (autolysis) and maintained the stability of the trypsin in solution. All enzyme immersion treatments were conducted at room temperature and pH was continuously monitored with a hand-held pH meter and pH-indicating strips.

Rinsing

Influenced by the results of a recent study that examined the effectiveness of removing amylase enzymes with water rinses alone (Erickson 1992), the following comparison of rinse baths was included. Two variations of rinsing procedures were followed in this study, each employing 10 ml of rinse water, but differing in time. Rinse 1 lasted for half an hour and Rinse 2 consisted of two half-hour immersions, where a fresh bath was supplied for the second half hour. The composition of the rinse water was dictated by the solution in which each enzyme was active. It is best to rinse initially under the same conditions minus the enzyme, so that denaturing of the enzyme will not take place while it possibly remains in the paper (fig. 3).

Drying

All samples were placed between polyester web and blotters and under glass and weights, and were allowed to dry over a period of seven days. This procedure is common practice within the paper conservation field and allows for equilibrium to be attained within the sheets, preventing planar distortions upon removal from the drying package.

MEASUREMENTS AND DATA

Sampling

A total of one hundred and twenty samples underwent enzyme treatments. Papers were cut into 3-cm squares. There were twenty samples per paper type per enzyme, with several variations of control samples. An HB graphite pencil was used to label each sample on the verso to assure that appropriate testing procedures were given to the correct sample. Throughout the evaluation processes, randomization of samples was conducted to avoid bias.

Paper samples were as homogeneous as possible. The sheets were large enough to accommodate the number of samples needed from each sheet (forty-six). Conscientious selection of paper dictated that each sheet would be identical in all its components including furnish, sizing, weight, and thickness. The only difference in the three sheets was their surface character.

Panel Assessment and Determination of Effectiveness of Treatment

The effectiveness of enzymes in removing protein adhesive was ranked according to original data based on human visual assessment. An eight-member panel consisting of paper conservators and conservation students judged the samples. While designing the experiment, the author believed that having the rare opportunity to utilize the eyes and minds of several trained professionals as well as those in training, could with their combined extensive experience in the field of paper conservation provide relevant and applicable information.

Fig. 3. Experimental rinsing procedure

The samples were laid out randomly and a survey form was generated to make documentation easy and consistent. Lighting conditions in which the panel participants viewed the samples were made as consistent as possible by using a Scandles light unit, whose bulb type measured 5300 Kelvin, in addition to natural light. All participants evaluated the samples from the same place in the paper conservation laboratory under the same conditions. Numerical codes were assigned to each sample to judge the effectiveness of the treatments: 4= excellent; 3=good; 2=fair; and 1=poor. Quantitative information was analyzed using averages and standard deviation based on the panel member's ranking system.

Microscopic Examination

Each sample was viewed in normal raking light under 6.5x magnification and evaluated for the enzyme's effectiveness at removing the protein adhesive. The adhesive residue was easily discernible by its characteristic pink tagging with the Rhodamine B isothiocyanate R-1755. A survey form similar to the one used for the panel assess-

ment was designed and used by the author to judge the effectiveness of treatments. Each sample was assigned a numerical code. Samples were examined in random order to avoid bias, quantitative information was averaged and charted, and standard deviation was calculated.

Measurement of Color Changes

A Minolta chromameter CR-221 with a Xenon light source was used on an area measuring 3 mm in diameter. Each sample was measured in four randomly chosen spots that were cut out of a Mylar template used for each sample. The findings were then averaged. The L*a*b* system was used to take into account perceived, not absolute, color differences. "L" measures light to dark; "a" measures red to green, and "b" measures yellow to blue. L-values proved to be the most useful, and numerical data were generated and charted employing averages and standard deviation.

Accelerated Aging Oven

Half of the samples underwent accelerated aging in a Blue M Electric AC Series Temperature/Humidity Chamber with MRS 7700 Control and were compared to the unaged samples. At this time no standards exist for the conditions that one should use when performing accelerated aging on paper artifacts. One of the most extensive investigations into the parameters one would use in accelerated thermal aging of paper with moisture recommends conditions of 70°C–80°C and 65% RH, and is endorsed by conservation scientist Robert Feller (Bansa and Hofer 1984; Feller 1994).

The MRS 7700 Controller is a circle-chart recorder that measures relative humidity. The instrument is capable of measuring, displaying, recording, and controlling relative humidity and/or temperature using dry- and wet-bulb temperatures. Conditions were set at 75°C and 65% RH and remained constant throughout the seven-day period.

A bookbinder's sewing needle was used to thread a piece of linen sewing thread through each of the 3-cm^2 paper samples so that they could be tied to the metal racks of the chamber without coming into contact with the metal.

RESULTS AND DISCUSSION OF EXPERIMENT

Panel Assessment

Overall, the results of the panel assessment show that both Pepsin 6887 and Trypsin 0134 worked well on all three paper types. Of the averaged one hundred twenty samples, 87% fell in the range of "good to excellent" and only 13% were given a rating in the "fair" range. None (0%) fell into the "poor" category.

An interesting trend was noted in comparing the results of the various rinsing procedures. In all cases, the averages given to the samples that were rinsed twice were higher than for those rinsed only once. However, this observation cannot necessarily be directly linked to the enzyme's activity. It may just be a case in which prolonged exposure to water may have assisted in the release of whatever little water-soluble adhesive remained in the support. Nevertheless, rinsing in two half-hour baths resulted in brighter papers, and that fact alone holds practical significance. According to the results of the panel assessment, no correlation was made between the effects of the different paper surfaces and the amount of adhesive removed.

Microscopic Examination

In evaluating the three pepsin-treated papers, the hot-press samples looked the best. Ninety-five percent of the hot press papers were given an excellent rating, while only 40% of the cold press and 25% of the rough surface papers were rated as excellent. These results could be interpreted to verify the theory that the topographical surfaces of the papers contribute to the effectiveness of this particular enzyme. The larger cavities existing on rough surface paper seem to either make it more challenging for the enzyme to get in and clip at these adhesive bonding sites or simply contain and hold larger amounts of adhesive, resulting in more adhesive residues. Examining the surfaces of the Trypsin 0134-treated samples showed no differences among any of the paper types. All samples were rated excellent because no adhesive residues were visually discernible.

Colorimetry

The evaluation of color measurements on the L-values read with the Minolta chromameter showed excellent results for both the enzymes employed in the study. Comparing the L-values of the three paper types before treatment with no adhesive to those after treatment, L-values had returned to within one integer of the control. L-value differences between the first and second rinse are relatively minute. One reason that little or no trends in the rinsing procedures were seen from these results could be due to the limitations of the area evaluated by the chromameter. Since there were only four openings on the template, approximately 12% of the entire 3-cm^2 sample was rated. During the course of the immersion treatments, it was observed that the samples had a tendency to float, especially from their edges, which resulted in some minor retention of adhesive on the edges of some of the samples.

Accelerated Aging

According to the L-values taken before and after aging, all samples were affected very little by the conditions of the temperature/humidity chamber. Differences in L-values before and after aging were on the order of 0.5. Specimens that were rinsed twice still exhibited brighter

values than those that were only rinsed once, but these differences in readings were very small as well. Statements cannot be made as to the degree of enzyme remaining in the papers and how they might change through accelerated temperatures and humidity. However, either the minor amounts of remaining adhesive or possible enzyme residues left in the papers showed no signs of rapid discoloration from the results obtained.

CONCLUSION OF EXPERIMENT

Clearly both trypsin and pepsin were capable of producing the desired effect of removing unwanted proteinaceous adhesives from all types of paper surfaces investigated. Both of the enzymes worked very well in room temperature conditions, yet the pH conditions were very different. If employing enzymes for treatment, test the pH of the paper and/or substrate being treated beforehand, as it is possible that the paper or substrate itself may act as a partial buffer or affect the enzyme's activity.

Not surprisingly there were significant visual benefits resulting in brighter papers from longer periods of rinsing, according to the panel assessment. Results obtained from the aged samples showed that no discernible color shifts had taken place over the course of one week under accelerated temperature and humidity conditions. The conservator must also balance the risk to the object when considering the length of immersion time that may be needed with lower concentrations. As long as *in situ* denaturation is avoided, concentrations in the range of 50–250 units of activity/ml result in very little, if any, detectable residue.

Certainly goals were met in finding an efficient and cost-effective enzyme. Additional concerns regarding enzyme retention or clearance from the artwork were greatly diminished when using such relatively small amounts of highly purified enzymes. Comparing the costs of two protease enzymes (Trypsin 0134 and formerly recommended Protease P5147) needed for a 1 l bath at 200 units of activity illustrates the clearly the more judicious choice:

Trypsin T 0134 (15,200 units/mg protein): 0.013 g needed, $1.34 total cost

Protease P 5147 (4 units/mg protein): 37.5 g needed, $1,683.00 total cost

Both trypsin and pepsin functioned well at room temperature though pH conditions were vastly different. Concerns for conservators regarding pH involve the potential solubility of media, shifting chemistry of pigments, and isoelectric equilibriums of binding media. Trypsin 0134 worked perfectly well in the neutral pH zone. Pepsin's activity curve ranges from pH 1–5 but this particular enzyme steadfastly operated within a pH range of 3.3–3.4. The conditions in which Pepsin 6887 operated are not traditionally ideal for practical use in paper conservation, yet there are always exceptions to general rules and appropriate conditions or circumstances may present themselves. In practice the needs of the object rather than the needs of the enzyme will always dictate treatment conditions.

CONSERVATION CASE STUDY

Background

The challenging and specific conservation needs of one particular object presented themselves in the summer of 2000. An Indian miniature painting attributed to one of the most famous painters in the Indian Rajput era, the artist Ebrahim, son of Ruknuddin, was under consideration for acquisition at The Metropolitan Museum of Art (TMMA) (fig. 4). Dating from circa 1675, the work, entitled *Khambhabati Ragini: A Page from a Dispersed Ragamala Series*, illustrates a ritual observance to the four-headed and -armed Hindu god Brahma. This god, according to Hindu mythology, was the creator and director of the universe.[6]

If acquired, the painting would be the first work by this artist to enter TMMA's collections and would join a small group of late seventeenth-century paintings from the Rajasthani state of Bikaner. Steven Kossak, Curator of

Fig. 4. Before treatment: Ebrahim, *Khambhabati Ragini: A page from a dispersed Ragamala series*, ca. 1675. Opaque watercolor and gold on paper. 16 x 13 cm, the Meteropolitan Museum of Art, 2000.321.

Southeast Asian Art, was very positive about the prospect of acquiring the painting but understandably troubled by the damages it had sustained. With its acquisition hinging on whether or not improvements could be made to its overall physical state, initial examination and micro-tests prompted the use of enzymes in the design phase of conservation treatment. However, limited by the painting's sensitivity to water, alternative methods of enzyme application would have to be found.

Indian miniatures are typically constructed of densely applied and burnished layers of transparent and opaque watercolor paint. This paint is applied over ink underdrawings on which a thin, white watercolor ground or priming is laid down on thick, multi-layered paper supports. The range of pigments and implements used to produce these pictures is relatively narrow. The extensive range of effects, including supremely delicate detail, results from a masterful handling of the materials, such as brushes, pens, and burnishers (fig. 5). Inorganic pigments, typically bound with gums, are highly valued for their good covering power, relative physical and chemical stability, and resistance to fading. With the exception of the inherent problems related to lead white, damages incurred in these paintings tend to be the result of external circumstances such as poor matting, framing, handling, neglect, or unfavorable environmental conditions.

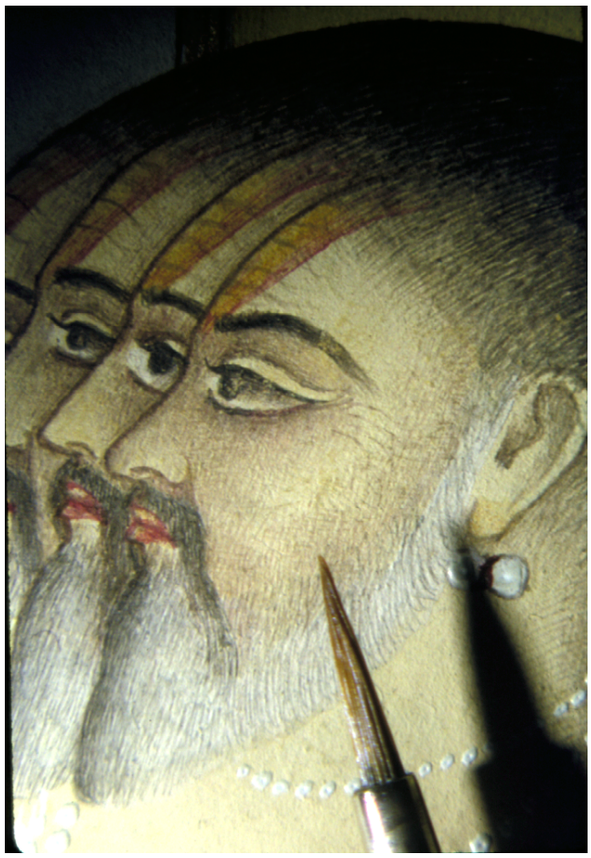

Fig. 5. Detail of TMMA 2000.321 with a 000 brush (10x magnification through binocular microscope).

Condition and Treatment Strategies

Measuring 16 x 13 cm, the object was mainly damaged by a former window mat composed of poorly processed wood pulp. The mat was applied directly on top of the painting perimeter with a thick, dark, globular layer of what seemed to be a protein adhesive. The evidence suggested that the window mat was separated subsequently from the painting in a brutal fashion, taking with it a good deal of the left edge, along with design material and primary support (fig. 4) All edges were left with a thick layer of adhesive residue with embedded mat fibers. There were areas that exhibited complete losses (left edge) and others in which in a pigment layer was covered and discolored with damage that was aesthetically unacceptable. A small sample of the adhesive was spot-tested with a 0.1% solution of fluorescamine in acetone, which confirmed that the adhesive was a protein, as it fluoresced blue-green in long-wave ultraviolet light. These results were confirmed with FTIR analysis (fig. 6).

After first consolidating the areas of the painting that were unstable (1% gelatin, brush-applied), the dense mass of bulky gray paper fibers was first softened and swelled by applying water in a controlled manner and removed with tweezers and microtools under binocular magnification. A fairly thick, gelatinous film on the surface of the water-sensitive paint layer remained. Although the paint film was not readily soluble in water, it would swell and become extremely vulnerable to any mechanical action.

Fig. 6. Detail of adhesive accretion with matboard paper fibers still embedded at upper right (8x magnification through binocular microscope). (TMMA 2000.321)

Keeping in mind the results of the initial tests and the vulnerabilities of the paint layer, the author devised a treatment strategy that eliminated any mechanical action near the painting's surface and instead involved the used of an enzyme incorporated into an agarose gel. A pure agarose that gelled at a low temperature was selected to be the carrier of the excellent-performing protease enzyme Trypsin T0134, the same one tested in the previous investigation. Unlike enzymatic immersion procedures in which the enzymes are freely mobile, topical applications involving surface-to-surface interactions require a relatively higher concentration of enzyme.[7]

Agarose

Agarose is a purified, linear, galactan hydrocolloid isolated from agar or agar-bearing marine algae. Structurally, it is a linear polymer consisting of alternating D-galactose and 3,6-anhydro-L-galactose units. Due to varying combinations of the 1,4-1,3 linkages in agarose, helixes occur in discrete regions of the chain. The helix-forming regions of agarose are the cross-linking points; polymer chains intertwine at the helixes. There is a strong intermolecular bonding between polymers at these points as well as hydrogen bonding between spirals in the helix regions of the chain itself. Currently there are at least twelve types of agaroses available commercially in a market driven by the DNA gene sequencing. Agarose Type VII, an agarose of low-gelling temperature, is excellent for in-gel enzymatic reactions. Gels exhibit excellent clarity and are available in an appropriate range of gel points (the temperature at which an aqueous agarose solution forms a gel as it cools).

The temperature/gelation relation is dependent upon the helix-forming regions of the polymer chains. Agarose, purchased in a powdered state, is added to water heated to 80°C–100°C. The heated water solvates the polymer chains. Separated, unfurled, and provided with sufficient energy to break bonds, the chains begin to cross-link at helix-forming regions upon cooling.

The concentration of agarose directly affects the extent of the cross-linking of the polymer chains, which in turn affects the rate of diffusion of water (or enzymes and water). Because there are greater numbers of chains in solution in gel preparations of higher concentrations, there are a greater number of helix-forming regions available for cross-linking. Cross-linking of chains creates a matrix through which water and enzymes diffuse. With greater cross-linking, the matrix is more extensive resulting in a smaller "pore size". The more extensive network slows diffusion because moisture (and the enzyme) has a greater distance to travel to the gel/substrate interface. Therefore, the rate of diffusion is slower in gels of higher concentration.

The gel can be prepared in varying concentrations depending on desired use. Concentrations are determined by weight-to-volume percentages of agarose to water. Generally, a weight-to-volume percentage of between 1.0–1.2 is useful for poulticing purposes or as a medium for enzymes. Under 1%, the gel may be too wet (adhesives may sink into the paper substrate), planar distortions may be a problem, and tidelines may occur in unwashed objects. Above 1.2%, the gel can be too rubbery and diffusion of moisture or enzyme may be too slow to be effective or useful. Experimentation with preparing the gel is essential in order to determine the concentration and consistency which might be appropriate for a given object or specific conservation problem.

Preparation of the Agarose/Enzyme Gel

A 1.0–1.2% gel is prepared by weighing out a certain amount of agarose, depending on concentration, and the water is heated to 80°C–100°C. The powdered agarose is then added and stirred until it is in solution; reheating may be necessary to dissolve clumps. If all of the agarose is not dissolved (undissolved agarose will look like small pieces of clear plastic floating in water), the result will be non-gelation, or a watery, turbid gel.[8] After the agarose is completely in solution, it is removed from the heat and allowed to cool (gelation occurs upon cooling). It takes approximately twenty to thirty minutes to set. As the temperature nears the gel point of the agarose, the pre-wetted enzyme is added at a temperature between 30°C–33°C (Type VII agarose's gel point is approximately 26±2°C at 1.5%) and then transferred to a small petri dish.[9] It is most convenient to fill the container to a depth of about 5–8 mm. Small, shallow, and smooth containers constructed from glass or polyethylene are the best containers for agarose.[10] Once in the container, the solution is swirled in a figure-eight pattern on the benchtop. Too much agitation may cause excessive aeration, which will either denature the enzymes or create unwanted obstacles within the gel matrix (fig. 7).

Moisture is an important factor in enzyme activity, as most enzymes require a moist environment in order to retain a proper configuration. Each enzyme has a unique sequence of amino acids and a unique configuration, which makes it a suitable catalyst for a specific substrate. Comprised of both hydrophobic and hydrophilic components, the enzyme orients itself in a unique configuration when placed in a moist environment. Hydrophobic portions fold into the interior while polar portions with the active sites are oriented to the exterior of the polymer. Without a moist environment, the enzyme unfolds, the unique configuration is lost, and the catalytic sites are destroyed.

Enzymes are capable of performing under non-ideal conditions although activity will be affected. At higher concentrations of agarose, moisture as well as enzyme at the gel/substrate interface is reduced. While the concentration of agarose may be varied, at higher concentrations, with

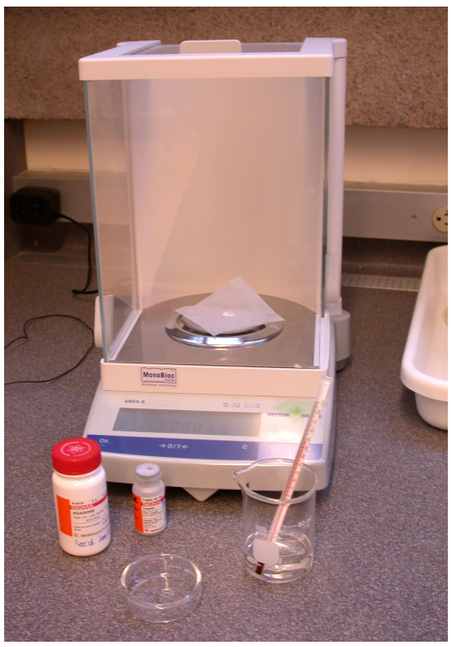

Fig. 7. Preparation of agarose/enzyme gel, weighing trypsin with a Mettler Toledo analytical balance (AB54-S).

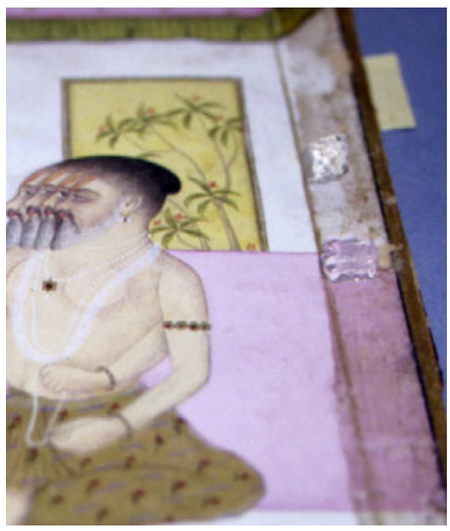

Fig. 8. Agarose gel blocks in place along right edge (TMMA 2000.321)

resulting restricted moisture, some enzyme chains may partially unfold. In addition, some chains may permanently denature, making fewer chains available for turnover. In the partially unfolded chains, contact of reacting substrates may be imperfect because of the altered configurations. Reactions may be possible, but slow.[11]

There are numerous advantages for conservators when using an agarose gel, which include: (1) the clear gel allows one to see substrate being acted upon, (2) minimal physical manipulation is necessary once *in situ*, (3) the concentration can be varied to alter wetness, (4) the gel keeps up to twenty-four hours with refrigeration, although reduction in activity has been noted; and (5) it can be tailored to a specific shape (as compared with other poultices used like methyl cellulose and hydroxy-propyl-methyl-cellulose). The 1.2% concentration of agarose was found to be a good working consistency in this particular case in that was not too wet and not too rubbery. It is probably best to err on the side of too wet so that diffusion of the enzyme can take place and one can easily wick excess moisture onto a piece of chromatography paper.

Small gel blocks were cut with a bamboo spatula and placed on the painting's surface—and the real excitement began (fig. 8). When viewed through the binocular microscope and focusing through the gel block to the surface of the painting, a beautiful swirling pattern of amber-colored adhesive could be seen moving within the block. The visuals were marvelous. The performance was ideal: fast moving ribbon-like patterns were swirling upwards, away from the object's surface into the gel block. Working in a systematic micro-layer progression, small gel blocks were selectively placed until all areas of adhesive accretions were successfully reduced (figs. 9a–b).

A note of caution is in order: although the enzyme gel eliminated the need for mechanical action, the five to eight minutes of interaction resulted in a swelled, moderately soft, and pliant paint surface and cellulose substrate. After gel blocks are removed, conservators must make empirical judgments to decide when to place the exposed areas under polyester web, blotters, and weights in order to

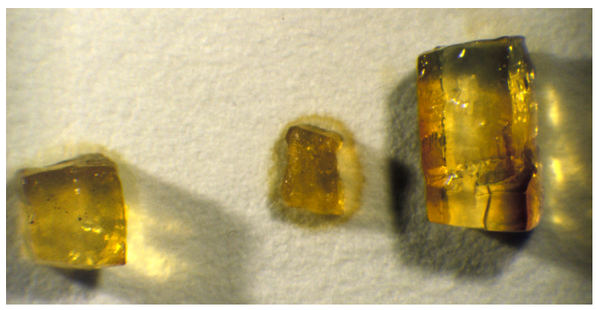

Fig. 9a. Detail of used agarose/enzyme gel blocks (7x magnification through binocular microscope).

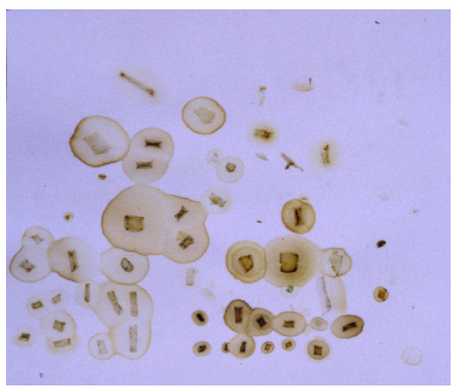

Fig. 9b. Agarose/enzyme gel blocks used in treatment

Fig. 10a. Before treatment, top half (TMMA 2000.321)

Fig. 10b. After treatment, top half (TMMA 2000.321)

Fig. 11a. Before treatment, upper left quadrant (TMMA 2000.321)

Fig. 11b. After treatment, upper left quadrant (TMMA.2000.321)

avoid unwanted planar distortions (figs. 10a–b). Other disadvantages may include the possibility of tidelines in unwashed objects or the dehydration of the gel before optimal enzyme action occurs.

Final phases of the conservation treatment consisted of tying the picture together physically and visually. Losses and skinned areas of the primary support were compensated with paper similar in furnish and texture and toned with Windsor & Newton artist's watercolors to match the background (figs. 11a–b). The treatment allowed the eye to focus on the all-important, central, and supreme god, Brahma (figs. 12a–b).

CONCLUSION

In this new millennium, we find ourselves in the golden age of enzyme technology. With discoveries and advances in isolating these proteins occurring at supersonic rates, we are endowed with a vast selection of highly purified enzymes that possess activities of up to twenty-thousand times that of just a decade ago. Enzymes and their specificity have and will continue to be important and powerful tools to the conservator, allowing us to treat objects otherwise untreatable. With an understanding of the technical and physical nature of the objects, and learning and borrowing from many disciplines, treatment strategies con-

Fig. 12a. Before treatment (TMMA 2000.321)

Fig. 12b. After treatment (TMMA 2000.321)

tinue to be shaped and refined to fit the needs of the unique and irreplaceable works of art in our care.

ACKNOWLEDGMENTS

Richard C. Wolbers, associate professor, Winterthur/University of Delaware Program in Art Conservation (WUDPAC); Janice Carlson, former senior scientist, Winterthur Analytical Laboratory; Harold M. Erickson, Conservation Department, Harry Ransom Humanities Research Center, University of Texas at Austin; Fran Mayhew, associate professor, University of Delaware; John Krill, head of Paper Conservation and adjunct professor, WUDPAC; Betty Fiske, paper conservator and adjunct professor, WUDPAC; Marjorie Shelley, conservator in charge, Sherman Fairchild Center for Works of Art on Paper and Photograph Conservation, the Metropolitan Museum of Art; Steven Kossak, curator of South and Southeast Asian Art, the Metropolitan Museum of Art.

NOTES

1. This investigation was developed and executed as part of graduate coursework at The Winterthur/University of Delaware Program in Art Conservation, 1998–99. It has previously been published in a slightly different form in *Art, Biology, and Conservation*, edited by Robert and Victoria Koestler. Yale University Press, 2004.

2. The Enzyme Data Bank site (release 35.0 June 2004, http://www.ebi.ac.uk/thornton-srv/databases/enzymes/) is an encyclopedic catalog of known isolated enzymes and includes extensive information on EC numbers, catalytic activities, and the latest discoveries. The ExPASy Molecular Biology Server (http://www.expasy.ch) is the home page of the Swiss Institute of Bioinformatics and contains numerous databases and protein analysis tools, as well as a host of links to other molecular biochemistry sites.

3. Trypsin consists of a single-chain polypeptide of 223 amino acid residues. Trypsin is a member of the serine protease family. The active site amino acid residues of trypsin include His 46 and Ser 183. Trypsin will cleave peptides on the C-terminal side of lysine and arginine amino acid residues. The rate of hydrolysis is slower if an acidic residue is on either side of the cleavage site. It will also hydrolyze ester and amide linkages of synthetic derivatives such as (BAEE) and (TAME).

4. Pepsin, unlike some other peptidases, hydrolyzes only peptide bonds, not amide or ester linkages. The cleavage specificity includes peptides with an aromatic acid on either side of the peptide bond, especially if the other residue is also aromatic or a dicarboxylic amino acid. Increased susceptibility to hydrolysis occurs if there is a sulfur-containing amino acid close to the peptide bond, which has an aromatic amino acid. Pepsin will

preferentially cleave at the carboxyl side of phenylalanine and leucine and to a lesser extent at the carboxyl side of glutamic acid.

5. Two recommended mid-range buffering compounds are TES, otherwise known as N-tris (hydroxymethyl)-methyl-2-aminoethanesulfonic acid, a structural analog to Trizma buffer; and one of the ethanesulfonic acid series of biological buffers developed to maintain midrange pKa and maximize water solubility while remaining chemically and enzymatically stable.

6. According to Hindu mythology, Brahma was the creator and director of the universe. He was the father of gods and humans alike and in classical Indian thought, he forms the trinity with Vishnu and Shiva representing a balancing and unifying force between their opposing energies. Brahma is often shown with four heads and four hands in which he hold the four Vedas, the holy scriptures of India. For as many heads that Brahma has, there are the same number of myths explaining why this is so. According to one myth Brahma produced the beautiful goddess Satuarupa from his own body. She was so lovely that he was unable to stop staring at her, and whenever she moved aside to avoid his gaze, he sprouted a new head in order that he might continue looking at her. Eventually, Brahma overcame his shyness and persuaded Satuarupa to marry him and they retired to a secret place for one hundred divine years at the end of which Manu, the first Man was born.

7. Concentrations of immersion versus topical applications are based empirically on surrogate trials, results during workshops, and actual treatments. They are as follows: 200–250 units/ml for immersions and 400–500 units/ml for gels.

8. If incompletely dissolved, the polymer chains will not have been completely dissociated. Turbidity is due to the optics of the coagulation of polymer chains. A watery gel or non-gelation is also the result of dissociated chains. The chains are not effectively separated so that there are fewer hydroxyl groups on the agarose available to hydrogen bond with water molecules.

9. When making gels, the author has found that prewetting the enzymes with 0.5–1.0 ml of deionized water assists in the reduction of electrostatic charges and ensures that more enzymes are actually incorporated into the mixture. The additional water should be added to the total volume calculations when making specific concentrations of agarose gels.

10. Only recently have other handling issues come to light. Common problems encountered within the research community relating to the loss of enzymatic activity are due to protein adsorption onto the surfaces of containers and pipette tips: ". . . proteins bind avidly to glass, quartz, and polystyrene surfaces. Hence containers made of these materials should not be used for enzyme samples. Containers and transfer devices constructed of low protein-binding materials such as polypropylene and polyethylene should be used whenever possible. . . ."

11. Another cause in the loss of enzyme activity, referred to as "spontaneous enzyme inactivation," is related to catalytic turnover. For some enzymes, the chemistry associated with turnover can lead to inactivation of the enzyme by covalent adduct formation, or by destruction of a key active site amino acid residue or cofactor. Free radicals build up during turnover, attacking the enzyme active site and rendering it inactive. Radical-based inactivation can be overcome with scavengers or dilution of enzyme concentration; minor adjustments in the concentration can sometimes ameliorate this situation.

PRODUCT INFORMATION

Paper

Watercolor paper (sample type 3): T. H. Saunders, English papermakers. Waterford series, mould-made from 100% cotton, 90 lb./ream, neutral pH, four deckles, watermarked, white.

Enzymes

Trypsin (EC 3.4.21.4), Type IX from porcine pancreas, T 0134, crystallized, dialyzed, and lyophilized; activity: 13,000–20,000 units per mg protein (Sigma Chemical Company).

Pepsin (EC 3.4.23.1), P 6887, crystallized and lyophilized; activity: 3,200–4,500 units per mg protein (Sigma Chemical Company).

Protein Tag

Rhodamine B isothiocyanate R-1755 (Sigma Chemical Colorado.)

REFERENCES

Andrews, T. 1990. An investigation into the removal of enzymes from paper following conservation treatment. *Book and Paper Group Annual* 9: 1–11

Bansa, H., and H. Hofer. 1984. Artificial aging as a predictor of paper's useful life. *Restaurator* 6: 21–60.

Burgess, H., and C. Charette. 1981. Aspects of image safety in the use of enzymes in paper conservation. In *International Council of Museums Committee for Conservation, 6th Triennial Meeting, Ottawa, Preprints,* 3: 10.1–10.13.

Copeland, R. 2000. *Enzymes: An introduction to structure, mechanism, and data analysis*. Canada: Wiley-VCH.

De Santis, P. 1983. Some observations on the use of enzymes in paper conservation. *Journal of the American Institute for Conservation* 23: 7–27.

Erickson, H. 1992. Usage recommendations for alpha-amylases: Maximizing enzyme activity while minimizing artifact binding residues. *Book and Paper Group Annual* 11: 24–33.

Faber, K. 1997. *Biotransformations in organic chemistry*. London: Springer: 50–67.

Feller, R. 1994. *Accelerated aging: Photochemical and thermal aspects*. Los Angeles: G. Paul Getty Trust.

Fletcher, S., and J. Walsh. 1979. The treatment of three prints by Whistler on fine Japanese paper. *Journal of the*

American Institute for Conservation 18(2): 118–126.
Grattan, D., J. Hillaire, H. Burgess, and J. C. McCawley. 1987. The characterization of enzymes for use in paper conservation. In *Conservation of library and archive materials and the graphic arts,* ed. G. Petherbridge, 15–24. London: Butterworth.
Hauser, R. 1987. Whaling museum restores fifty logbooks. *The Abbey Newsletter* 11(5): 76–77.
Mills, J., and R. White. 1994. *The organic chemistry of museum objects*. London: Butterworth.
Pacific Northwest National Laboratory. 2002. Enzyme discovery to benefit homeland security, industry. Department of Energy, http://www.eurekalert.org/pub_releases/2002-09/dnnl-edt091202.php.
Rickman, C. 1988. Wallpaper conservation: Conservation today. In *Preprints of the UKIC 30th anniversary conference*, 64–70. London: United Kingdom Institute of Conservation.
Segal, J., and D. Cooper. 1977. The use of enzymes to release adhesives. *The Paper Conservator* 2: 47–50.
Shelley, M. 1980. The conservation of the van Rensselaer wallpaper. *Journal of the American Institute for Conservation* 20: 1–4.
Sipos, T., and J. R. Merkel. 1970. An effect of calcium ions on the activity, heat stability, and structure of trypsin. *Biochemistry* 9(14): 2766–2775.
Tse, S., and H. Burgess. 1988. Effects of enzyme treatment on the degradation of paper. In *Symposium '88 abstracts.* Ottawa: Canadian Conservation Institute.
van der Reyden, D. 1988. Technology and treatment of a folding screen: Comparison of oriental and western techniques. In *Preprints of the contributions to the Kyoto Congress: The conservation of Far Eastern art,* eds. J. Mills, P. Smith, and K. Yamasaki, 64–69. London: International Institute for Conservation.
Walsh, K. A.1970. Trypsinogens and trypsins of various species. *Methods in Enzymology* 19: 41–63.
Wendelbo, O. 1974. Extraction of papyri from gesso cartonnage: A new method based on enzymatic approach. *Proceedings from the 14th international conference of papyrology*, 41–52. Oxford.

YANA VAN DYKE
Assistant Conservator
Sherman Fairchild Center for Works of Art on Paper and Photograph Conservation
The Metropolitan Museum of Art
New York, New York
yana.vandyke@metmuseum.org